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Transcriptional profiling of Marek's disease virus genes during cytolytic and latent infection

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Abstract Marek's disease (MD), a lymphoproliferative disease of chicken is caused by a highly cell-associated α-herpesvirus, Marek's disease virus (MDV). MDV replicates in chicken lymphocytes and establishes a latent infection within $CD4^+$ T cells. The expression analysis of limited viral transcripts have revealed differences in gene expression pattern during cytolytic and latent phases of MDV infection. In this study, we conducted a global gene expression profiling of MDV using oligonucleotide-based Affymetrix GeneChip Chicken Genome Arrays. These arrays contain probe for more than 32,000 chicken transcripts and most of the known MDV genes and open reading frames. Two-week-old MD-susceptible chickens were inoculated with an oncogenic strain of MDV, and spleen samples were collected 5 and 15 days post inoculation (cytolytic and latent infection, respectively) for RNA isolation and microarray analysis. Array results displayed a significant differential pattern of viral transcriptome between the two phases of MDV infection. The expression levels of more than 78 MDV genes were increased during the cytolytic infection when compared to latent infection (2–11-fold increase). A 23-KD nuclear protein, meq oncoprotein, and R-LORF5 were among the few viral genes that were expressed during both phases of infection. In addition, there were at least 11 known and hypothetical genes that had no significant transcriptional activities during either stages of infection. These chicken genome arrays have considerable promise, as a valuable tool in understanding the molecular mechanism regulating MDV cytolytic and latent infection, and providing insights into the chicken gene expression pattern and associated biological pathways in response to different phases of viral pathogenesis.

Keywords Microarray · Gene expression · RT-PCR · Herpesvirus · Cytolytic · Latency

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Introduction

Marek's disease virus (MDV) is an oncogenic cell-associated α-herpesvirus that causes lymphoproliferative disease in the domestic chickens resulting in T-cell lymphomas in visceral organs and peripheral nerves as early as 3–4 weeks post infections [1–3]. A major burst of replication in B lymphocytes, the primary target cells for cytolytic infection, occurs between 3- and 6-day post infection (dpi), which is followed by attraction and subsequent infection of thymus-derived $CD4^+$ T cells at about 7 dpi. During this second phase of infection, viral replication is decreased and a latent phase is established within the activated $CD4^+$, which lasts up to 2 weeks prior to reactivation [2, 4]. The latently infected T lymphocytes are the means of virus dissemination to the skin and feather

follicle epithelial cells, the only anatomical site for production and release of infectious-enveloped virus particles in the infected chickens [5–7]. A subset of latently infected transformed $CD4^+$ T cells that harbor MDV genome, migrate, and establish lymphomas in the skin, peripheral nerves, and visceral organs [2, 7].

The MDV was initially classified as a γ-herpesvirus according to its biological properties. However, based on microscopic studies, restriction enzyme analysis, and DNA sequence homology, MDV genome is now classified as an α-herpesvirus [8–11]. MDV genome is about 180 Kb in length and encodes for more than 100 genes [11–15]. The complete nucleotide sequences of all three MDV serotypes have been recently published [11, 13, 16, 17]. Sequence analysis has revealed that overall MDV genome structure resembles those of human herpesvirus 1 and 3 (herpes simplex virus type 1 (HSV1), varicella-zoster virus (VZV), respectively) with a long and a short unique region (UL, US) and flanking inverted repeat sequences TR_L/IR_L and TR_S/IR_S, respectively [8].

The unique long and short regions of MDV genome are generally conserved and collinear with the corresponding regions in other herpesviruses. MDV-specific genes, however, are located predominantly in the TR_L and IR_L repeat regions [18]. As this is the case with other herpesviruses, three phases of gene expression pattern are recognized in MDV infection with transcripts falling in the category of immediate-early, early, and late kinetic classes [19]. The transcriptional activities of most genes that are expressed in lytic infection are suppressed during the latent phase of MDV infection [20]. Of the limited number of genes expressed during the latent infection, the latency-associated transcripts (LATs) that map antisense to MDV ICP4 gene were identified and characterized [21–23]. These transcripts consist of two small RNAs, several spliced variants, and a 10 Kb long RNA that spans the entire ICP4 coding region [24–26]. Meq, a homologue of *fos* and *jun* oncogenes [27], is also expressed during the latent infection [28]. In addition to playing a major role in T-cell transformation, meq has also been shown to block apoptosis of latently infected cells and transactivate gene expression depending on its dimerization partners and MDV genome binding sites [29, 30].

Although microarray analysis of chicken genes in response to MDV and herpesvirus of turkey (HVT) infection have been previously investigated [31–33], a comprehensive MDV gene expression profiling has not been studied before and the potential involvement of specific viral genes in the initiation and maintenance of different phases of infection remains unknown. It is speculated that gene expression pattern between lytic and latent infection is regulated by transcriptional activities of genes within the flanking IR_L repeat region of MDV genome [34].

Although vaccination protects against mortality, clinical signs, and lymphoma formation, it does not provide protection against infection, replication, and shedding of challenge viruses. Consequently, super-infection of vaccinated birds with new strains of MDV with increased virulence has been continuously observed in the field [35, 36]. Cytolytic infection and establishment of latency are two critical aspects of MDV pathogenesis. Identification and characterization of specific genes associated with either of these steps would provide insights into the molecular mechanism of pathogenesis, host-pathogen interaction, and eventual control of MDV infection. In order to this end, we have conducted a global gene expression profiling of MDV in susceptible chickens using Affymetrix GeneChip Chicken Genome Arrays. Up to our knowledge, this is the first comprehensive comparative MDV gene expression analysis during cytolytic and latent infection.

Materials and methods

Experimental chickens

Chickens were F_1 progeny ($15I_5X7_1$) of Avian Disease and Oncology Laboratory (ADOL) Line $15I_5$ males and 7_1 females. The $15I_5X7_1$ birds were from unvaccinated breeder hens and carried no maternal antibodies to MDV or HVT. Chicks were hatched at ADOL poultry facility and housed in modified Horsfall-Bauer isolation units for the duration of the experiment.

Virus

A very virulent plus (vv+) strain of MDV, 648A-p8 (passage 8), which is propagated and maintained in our laboratory, was used in this experiment [37].

RNA extraction and array processing

Total RNA was isolated from the spleen tissues of MDV-inoculated and control birds by TRIzol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Residual DNA was digested by DNase treatment of RNA samples using Ambion's RNase-free DNase kit (Austin, TX). In order to prove that all DNA contamination was digested by DNase treatment, the RNA samples were subjected to PCR analysis without reverse transcription (RT) reaction. The PCR analysis failed to amplify chicken glyceraldehyde 3-phosphate dehyrogenase (GAPDH) amplicon after 35 cycles of reaction (data not shown). The

DNase-treated samples were used for array and RT-PCR analysis only after this vigorous testing. The cDNA synthesis, labeling, hybridization, and scanning process were all carried out at the Research Technology Support Facility (RTSF) of Michigan State University in East Lansing, Michigan, according to the protocol provided by Affymetrix. Raw data was provided as CEL or Excel files for statistical analysis.

Real-Time RT-PCR

Real-Time RT-PCR analysis of relative quantification of a selected MDV genes was carried out at RTSF of Michigan State University. Briefly, 2.5 µl of 1/20 dilution of the oligo dT-based RT product from 4 µg of total RNA was used for each reaction. A batch of 300 nM of each for specific sense and anti-sense primers were used in the presence of 5 µl SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The amplification program was as follows: 50°C 2 min, 95°C 10 min, and 40 cycles at 95°C for 15 s followed by 58°C for 1 min. All the reactions were run in duplicates in a 7900HT Sequence Detection System (Applied Biosystems). The primers for MDV and chicken genes were designed using MacVector software (Accelrys, San Diego, CA). All the primers were synthesized by Operon Biotechnologies, Inc (Huntsville, Alabama). The primer sequences and their amplicons are listed in Table 1. The relative quantification of the MDV genes was determined using $2^{-\Delta\Delta CT}$ method [38]. The expression level of each gene was normalized to that of chicken GAPDH (Table 2).

Experimental design

One-day-old chicks were randomly distributed into 2 groups of 24 birds each in separate isolators (A and B). Birds in groups A were inoculated subcutaneously with 10,000 plaque-forming units (PFU) of 648A-p8 MDV at 2 weeks of age. Birds in group B served as negative non-inoculated controls. Three birds from each of the infected and control groups were euthanized by CO_2 inhalation and necropsied at 5 and 15 dpi (cytolytic and latent infection, respectively). The spleen tissues were collected and immediately stored in RNAlater (Ambion, Austin, TX). Total RNA was isolated from the six collected spleen tissues as described above and equal amount of each sample was individually examined by array analysis.

Statistical analysis

The analysis of differential gene expression requires normalization within and between arrays to remove technological sources of variations. The normalization estimate of microarray data was based on a set of host-encoded housekeeping genes. These were triplicate spots of β-actin, GAPDH, and eukaryotic elongation factor 1 α 1 (EEF1α1). Since viruses lack an appropriate control or

Table 1 Primers used for validation of microarray data analysis

Name	Direction	Sequence	Product (bp)
vIL8	Forward	5′-AGGTATGTGTGGACCCTGAGGC-3′	78
	Reverse	5′-CCTTCCTTGTGCGATGCTGTC-3’	
PP38	Forward	5′-AAGGGTGATGGGAAGGCGATAG-3’	128
	Reverse	5′-GCATACCGACTTTCGTCAAGATG-3′	
UL39	Forward	5′-TATGGACCCTGTTTGACTCCCG-3′	169
	Reverse	5′-AATGGACTTCCTGTAGAGGCTGC-3′	
UL41	Forward	5′-AATCTTTACGCCCCCCTCG-3′	161
	Reverse	5′-GTCTTCAACTGCTGTCTCATCACG-3′	
UL49.5	Forward	5′-GCTATTTTGATTGCCACTTCGCA-3′	163
	Reverse	5′-AGATTACCGCCACCAGACCC-3′	
Meq	Forward	5′-AAGTCACGACATCCCCAACAGC-3′	142
	Reverse	5′-TACATAGTCCGTCTGCTTCCTGCG-3′	
Meq/R-LORF5[a]	Forward	5′-GCAGACGGACTATGTAGA-3′	~300
	Reverse	5′-CTGCTTTATCAAGACTCG-3′	
gB	Forward	5′-CGGTGGCTTTTCTAGGTTCG-3′	68
	Reverse	5′-CCAGTGGGTTCAACCGTGA-3′	
GAPDH	Forward	5′-TGCCATCACAGCCACACAGAAG-3′	123
	Reverse	5′-ACTTTCCCCACAGCCTTAGCAG-3′	

[a] Forward primer is located within meq and reverse primer within R-LORF5

Table 2 Relative fold difference in MDV gene expression using comparative C_T method

Genes	Days post infection	C_T Mean ± SD	ΔC_T ± SD	$\Delta\Delta C_T$ ± SD	Fold difference
vIL8	5	15.15 ± 0.77	2.33 ± 0.82	−6.48 ± 0.82	89
vIL8	15	25.73 ± 0.45	8.81 ± 0.57	6.48 ± 0.57	
pp38	5	16.72 ± 0.74	3.9 ± 0.79	−6.49 ± 0.79	90
pp38	15	27.30 ± 0.67	10.39 ± 0.75	6.49 ± 0.75	
UL39	5	17.63 ± 0.98	4.81 ± 1.02	−7.54 ± 1.02	186
UL39	15	29.27 ± 0.74	12.35 ± 0.82	7.54 ± 0.82	
UL41	5	19.62 ± 0.35	6.8 ± 0.44	−7.18 ± 0.44	145
UL41	15	30.90 ± 0.88	13.98 ± 0.94	7.18 ± 0.94	
UL49.5	5	22.53 ± 1.04	9.71 ± 1.08	−7.79 ± 0.08	221
UL49.5	15	34.42 ± 0.45	17.5 ± 0.57	7.79 ± 0.75	
Meq	5	21.27 ± 0.27	8.45 ± 0.39	1.51 ± 0.39	
Meq[a]	15	23.86 ± 0.31	6.94 ± 0.47	−1.51 ± 0.47	2.8
Meq/RLORF5	5	30.96 ± 0.39	18.14 ± 0.48	3.62 ± 0.48	
Meq/RLORF5[a]	15	31.44 ± 0.62	14.52 ± 0.71	−3.62 ± 0.48	12
gB	5	18.95 ± 0.93	6.13 ± 0.97	−7.4 ± 0.97	169
gB	15	30.45 ± 1.03	13.53 ± 1.09	7.4 ± 1.09	

[a] For meq and meq/RLORF5 with higher ΔC_T values at 15 dpi, the fold differences was calculated using 5 dpi as the reference or base line. The fold differences for the rest of the samples were calculated using 15 dpi as the base line

"housekeeping" gene to be used in statistical analysis, using host-encoded housekeeping genes for normalization of viral gene expression is a common practice in microarray studies [39–47]. The data was normalized by the publicly available software R v.2.4.1 using gcrma [48] followed by linear model analysis to identify differentially expressed genes between 5 and 15 dpi. The list of genes differentially expressed was ranked based on the moderated *t*-statistics introduced by Smyth [49]. In this approach, a gene-wise linear model is fitted based on the experimental design. For each gene, a moderated *t*-statistic is calculated using posterior standard deviation to extract those with maximal differential expression. The *P*-values generated by this analysis were adjusted for multiple comparisons to control the false discovery rate (FDR), which is the expected proportion of false discoveries among the rejected hypotheses. The boxplot depicts the average of the log-normalized gene expression between the two phases of infection (Fig. 1). The volcanoplot arranges genes along the dimensions of biological and statistical significance. The higher values on the *y*-axis indicate statistically significant expression values (Fig. 2).

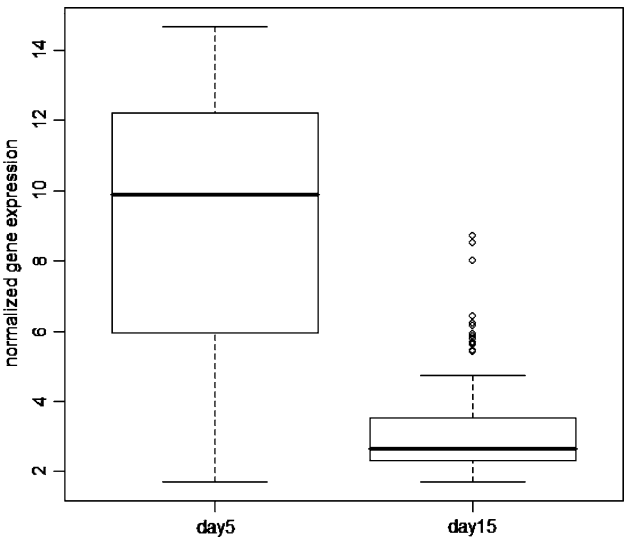

Fig. 1 Box Plot of microarray validation. This plot depicts the overall log-transformed signal intensities of spotted probes for MDV genes during the cytolytic and latency infection (5 and 15 dpi, respectively). This comparative gene expression profiling is a statistical representation of gene expression pattern and a validation for microarray data. The line inside the box represents the median value

Results

A comparative global gene expression profiling was conducted between the cytolytic and latent infection of MDV. Selection of the birds for microarray analysis was random for lytic phase and based on clinical sign (transient paralysis, depression, and crippling) for latent infection. The spleen tissues from three MDV-infected and three control chickens at 5 and 15 dpi were used for RNA isolation and array analysis (three biological replications). The RNA samples of the control birds used in the study were for detection of background signal intensity (noise) in the array analysis. The samples from the latent infection served as the baseline in all statistical analysis.

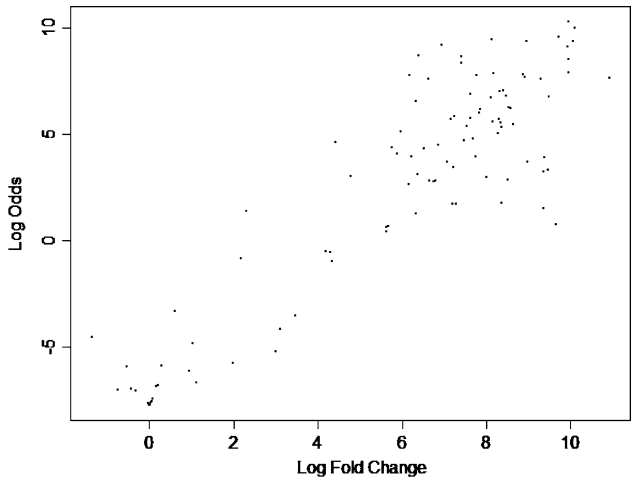

Fig. 2 Volcanoplot of microarray validation. This plot arranges genes along dimensions of biological and statistical significance. The horizontal axis is the log fold change (base 2) between two phases of infection with 15 dpi being the baseline. More significant fold changes appear higher while the fold change axis indicates biological impact. As the plot indicates, most genes are upregulated in lytic infection while only a few genes are probed during the latency infection (log fold change <0)

Gene expression profiling

Figure 1 shows the overall signal intensities of array data as box plot for both lytic and latent infection. The plot shows a comparative numerical and statistical expression profiling of MDV genes during both phases of infection and provides a general validation for the array data by depicting higher expression values for MDV genes in lytic phase than those of the latent infection. Figure 2 is a volcano plot depicting fold change versus statistical significance. The horizontal dimension is the log fold change (base 2) between the two groups with day 15 as a baseline and the vertical axis representing the B-statistic [50] based on an empirical Bayes method for analyzing replicated microarray data. More significant fold changes appear higher while the fold change axis indicates biological impact. As the plot indicates, most genes are upregulated in lytic infection, while only a few genes are probed during the latency (log fold change <0).

Table 3 depicts the normalized expression profiles of MDV genes during both phases of infection. For comparative analysis, the data from the cytolytic infection was compared to that of latent infection as a baseline. A total of 79 genes were upregulated ($\sim$2–11-fold) during the lytic infection in comparison to latent infection. There were no significant changes in the expression levels of 11 known and hypothetical genes at both phases of infection (Table 3, middle section). The fluorescence signal intensities of these genes were at the levels of background noise seen in the control non-infected chickens. There were only three genes that had higher expression levels during latency when compared to lytic cycle (Table 3, lower section).

Real-Time RT-PCR validation of array data

Table 2 depicts the Real-Time PCR analysis of selected MDV genes that were differentially expressed during the lytic and latent infection. The array data reveals that vIL8, pp38, UL39, UL41, UL49.5, and glycoprotein B (gB) were among the many genes that were significantly upregulated in the cytolytic infection. The changes in the expression level of meq oncogene were minimal. Meq/R-LORF5 splice variant, however, was expressed at higher level in latent infection in comparison to the lytic phase. The data obtained from the Real-Time PCR analysis is in good agreement with the array results. The meq gene expression level was 2.8× higher during the latent phase in comparison to the lytic phase of infection. According to the array data, R-LORF5 was the only gene with statistically significant higher expression level in the latent phase of MDV infection (P value = 0.035). This observation is confirmed by the Real-Time RT-PCR results (12-fold difference).

Discussion

MDV is an oncogenic α-herpesvirus that causes various clinical syndromes including T-cell lymphomas in the chickens. In order to provide insights into the molecular mechanism of virus pathogenesis and to better understand the patterns of viral gene transcription, we conducted a microarray experiment using Affymetrix chicken genome arrays. Although, these DNA chips contain probes for both the chicken and MDV genes, here we only analyzed the magnitude of expression and relative abundance of virus genes. The expression analysis of the chicken genes in response to MDV infection will be reported later.

In order to verify that 5 and 15 dpi represent cytolytic and latent infection, respectively, the expression pattern of selected MDV genes was analyzed. Real-Time RT-PCR analysis revealed that meq, an MDV-encoded oncoprotein, and meq/RLORF5 are expressed in both phases of infection (Table 2, minor changes in C_T values). Meq is a homologue of *fos* and *jun* oncogenes with anti-apoptotic property and is required for tumor development and maintenance [52]. Anti-apoptotic property of meq is probably a viral strategy to evade destruction of MDV-infected cells by cellular apoptosis that is critical for viral growth. Functional studies of a meq-deleted mutant virus, however, suggest that meq is involved but not essential for latency [53]. In contrast to meq and meq/RLORF5, the transcriptional activities of vIL-8, pp38, UL39, UL41, UL49.5, and

Table 3 Comparative MDV gene expression profiling during cytolytic and latency infection

	Probe ID	Gene ID	Fold change	Adjusted *P* Value
1	AF243438.CDS90.S1_s_at	US1, phosphoprotein ICP22-like protein (MDV088)	10.91	1.91E-06
2	AF243438.CDS35.S1_s_at	UL22, envelope glycoprotein H-like protein (MDV034)	10.1	3.63E-07
3	AF243438.CDS62.S1_s_at	UL48, **L**[a], gene transactivator-like protein (MDV061)	10.05	5.02E-07
4	AF243438.CDS15.S1_s_at	UL2, uracil DNA glycosylase-like protein (MDV014)	9.96	1.03E-06
5	AF243438.CDS16.S1_s_at	UL3, nuclear phosphoprotein-like protein (MDV015)	9.95	3.14E-07
6	AF243438.CDS63.S1_s_at	UL49, **L**, tegument phopsphoprotein-like protein (MDV062)	9.95	1.91E-06
7	AF243438.CDS6.S1_s_at	R-LORF-10, 14 KD lytic phase protein	9.94	5.62E-07
8	AF243438.CDS69.S1_s_at	UL54, **IE**[b], post translational gene regulation, ICP27 (MDV068)	9.72	5.02E-07
9	AF243438.CDS25.S1_s_at	UL12, DNase-like protein (MDV024)	9.65	4.00E-04
10	AF243438.CDS10.S1_s_at	R-LORF-14, **E**[c], 24 KD phosphoprotein, pp24 (MDV008)	9.48	3.94E-06
11	AF243438.CDS71.S1_s_at	UL55-like protein (MDV070)	9.46	5.25E-05
12	AF243438.CDS98.S1_s_at	US8, membrane glycoprotein E-like protein (MDV096)	9.37	3.36E-05
13	AF243438.CDS3.S1_s_at	CxC chemokine, vIL8	9.36	5.58E-05
14	AF243438.CDS31.S1_s_at	UL18, nucleocapsid protein-like protein (MDV030)	9.35	2.18E-04
15	AF243438.CDS70.S1_s_at	LORF4 (MDV069)	9.3	1.91E-06
16	AF243438.CDS57.S1_s_at	UL43, probable membrane protein (MDV056)	8.97	3.93E-05
17	AF243438.CDS17.S1_s_at	UL4, nuclear protein-like protein (MDV016)	8.94	5.02E-07
18	AF243438.CDS23.S1_s_at	UL10, viron membrane glycoprotein M-like protein (MDV022)	8.91	1.91E-06
19	AF243438.CDS48.S1_s_at	UL34, membrane phosphoprotein-like protein (MDV047)	8.88	1.91E-06
20	AF243438.CDS21.S1_s_at	UL8, DNA helicase-primase associated protein-like protein (MDV020)	8.63	1.01E-05
21	AF243438.CDS97.S1_s_at	US7, membrane glycoprotein I-like protein (MDV095)	8.58	6.27E-06
22	AF243438.CDS13.S1_s_at	LORF2 (MDV012)	8.52	6.00E-06
23	AF243438.CDS85.S1_s_at	RS1, immediate-early gene transactivator ICP4-like protein (MDV084)	8.52	7.36E-05
24	AF243438.CDS18.S1_s_at	UL5, DNA helicase-primase associated protein-like protein (MDV017)	8.5	3.94E-06
25	AF243438.CDS46.S1_s_at	UL32, DNA packaging protein-like protein (MDV046)	8.41	3.33E-06
26	AF243438.CDS32.S1_s_at	UL19, major capsid protein-like protein (MDV031)	8.36	1.12E-05
27	AF243438.CDS28.S1_s_at	UL15, DNA packaging protein-like protein	8.31	3.42E-06
28	AF243438.CDS44.S1_s_at	UL30, DNA polymerase catalytic subunit-like protein (MDV042)	8.3	8.69E-06
29	AF243438.CDS36.S1_s_at	UL23, thymidine kinase-like protein (MDV036)	8.27	1.43E-05
30	AF243438.CDS49.S1_s_at	UL35, capsid protein-like protein (MDV048)	8.18	1.91E-06
31	AF243438.CDS56.S1_s_at	UL42, DNA polymerase processivity subunit-like protein (MDV055)	8.14	9.40E-06
32	NC-002229.CDS44.S1_s_at	UL26, capsid maturational protease, scaffold protein (MDV038)	8.12	5.02E-07
33	AF243438.CDS41.S1_s_at	UL27, virion membrane glycoprotein B-like protein (MDV040)	8.11	3.96E-06
34	AF243438.CDS24.S1_s_at	UL11, myristylated tegument protein-like protein (MDV023)	8	6.58E-05
35	AF243438.CDS20.S1_s_at	UL7-like protein (MDV019)	7.85	6.32E-06
36	AF243438.CDS27.S1_s_at	UL14, minor tegument protein-like protein (MDV026)	7.83	7.09E-06
37	AF243438.CDS60.S1_s_at	UL46, **L**, tegument phosphoprotein-like protein (MDV059)	7.77	1.91E-06
38	AF243438.CDS52.S1_s_at	UL38, capsid protein-like protein (MDV051)	7.63	3.76E-06
39	AF243438.CDS65.S1_s_at	UL50, dUTPase-like protein (MDV063)	7.62	8.60E-06
40	AF243438.CDS96.S1_s_at	US6, membrane glycoprotein D-like protein (MDV094)	7.52	1.11E-05
41	AF243438.CDS66.S1_s_at	UL51, virion phophoprotein-like protein (MDV065)	7.42	9.47E-07
42	AF243438.CDS72.S1_s_at	LORF10, **IE** (MDV071)	7.41	1.22E-06
43	AF243438.CDS53.S1_s_at	UL39, ribonucleotide reductase large subunit-like protein (MDV052)	7.28	1.83E-04
44	AF243438.CDS19.S1_s_at	UL6, minor capsid protein-like protein (MDV018)	7.24	8.39E-06
45	AF243438.CDS14.S1_s_at	UL1, virion surface glycoprotein L-like protein (MDV013)	7.21	4.76E-05
46	AF243438.CDS43.S1_s_at	UL29, single stranded DNA binding protein-like protein (MDV042)	7.19	1.82E-04
47	AF243438.CDS74.S1_s_at	R-LORF-14A, **E**, 38KD phophoprotein, pp38 (MDV073)	7.15	8.69E-06
48	AF243438.CDS12.S1_s_at	Hypothetical protein	7.1	3.93E-05

Table 3 continued

	Probe ID	Gene ID	Fold change	Adjusted *P* Value
49	AF243438.CDS59.S1_s_at	UL45, envelope/transmembrane protein-like protein, cell fusion (MDV058)	6.93	5.61E-07
50	AF243438.CDS93.S1_s_at	US2, virion protein (MDV091)	6.85	2.17E-05
51	AF243438.CDS61.S1_s_at	UL47, **L**, tegument phosphoprotein-like protein (MDV060)	6.79	7.49E-05
52	AF243438.CDS58.S1_s_at	UL44, virion membrane glycoprotein C-like protein (MDV057)	6.75	7.50E-05
53	AF243438.CDS95.S1_s_at	SORF4 (MDV093)	6.64	7.43E-05
54	AF243438.CDS34.S1_s_at	UL21, tegument protein-like protein (MDV033)	6.63	1.91E-06
55	AF243438.CDS73.S1_s_at	LORF5 (MDV072)	6.51	2.38E-05
56	AF243438.CDS55.S1_s_at	UL41, virion host shutoff protein-like protein (MDV054)	6.38	9.47E-07
57	AF243438.CDS92.S1_s_at	SORF3 (MDV090)	6.37	6.07E-05
58	AF243438.CDS91.S1_s_at	US10, capsid/tegument associated phosphoprotein-like protein (MDV089)	6.34	4.75E-06
59	AF243438.CDS38.S1_s_at	UL25, DNA packaging-like protein (MDV037)	6.32	2.63E-04
60	AF243438.CDS47.S1_s_at	UL33, DNA packaging (MDV045)	6.22	3.30E-05
61	AF243438.CDS50.S1_s_at	UL36, large tegument protein-like protein (MDV049)	6.17	1.91E-06
62	AF243438.CDS22.S1_s_at	UL9, ori binding protein-like protein (MDV021)	6.16	8.36E-05
63	AF243438.CDS11.S1_s_at	Lipase	5.97	1.33E-05
64	AF243438.CDS86.S1_s_at	Antisense RNA protein (Similar to HSV1 RS1)	5.89	2.97E-05
65	AY510475.CDS81.S1_s_at	UL56, membrane protein	5.76	2.38E-05
66	AF243438.CDS84.S1_s_at	Hypothetical protein (MDV082)	5.66	4.22E-04
67	AF243438.CDS94.S1_s_at	US3, serine therionine protein kinase-like protein (MDV092)	5.64	5.16E-04
68	AF243438.CDS68.S1_s_at	UL53, IE, glycoprotein K-like protein (MDV067)	5.63	4.39E-04
69	AF243438.CDS42.S1_s_at	UL28, DNA packaging protein-like protein (MDV041)	4.79	6.58E-05
70	AF243438.CDS8.S1_s_at	R-LORF12 (MVD007)	4.42	1.93E-05
71	AF243438.CDS64.S1_s_at	UL49.5, L, envelope/tegument protein-like protein (MDV064)	4.33	1.60E-03
72	AF243438.CDS67.S1_s_at	UL52, DNA helicase/primase-like protein associated protein (MDV066)	4.28	1.16E-03
73	AF243438.CDS99.S1_s_at	SORF2 (MDV087)	4.19	1.12E-03
74	AF243438.CDS33.S1_s_at	UL20, transmembrane protein-like protein, virus egress (MDV032)	3.46	0.0148
75	AF243438.CDS99.S1_at	SORF2-like (MDV097)	3.1	0.02521
76	AF243438.CDS29.S1_s_at	UL16, tegument protein-like protein (MDV028)	3	0.06273
77	AF243438.CDS88.S1_s_at	Cytoplasmic protein (MDV086)	2.29	0.00024
78	AY510475.CDS10.S1_s_at	R-LORF-11	2.16	0.00146
79	AF243438.CDS37.S1_s_at	UL24, nuclear protein (MDV035)	1.98	0.10324
80	AF243438.CDS30.S1_s_at	UL17, tegument/minor capsid protein-like protein (MDV029)	NC[d]	0.24435
81	AF243438.CDS9.S1_s_at	Hypothetical protein	NC	0.04552
82	AY510475.CDS8.S1_s_at	R-LORF8	NC	0.11516
83	NC-002229.CDS58.S1_at	LORF7 (MDV050)	NC	0.28468
84	AY510475.CDS6.S1_s_at	R-LORF3	NC	0.66769
85	AF243438.CDS87.S1_s_at	Hypothetical protein (MDV085)	NC	0.76897
86	AF243438.CDS2.S1_s_at	Arg-rich protein	NC	0.97909
87	AF243438.CDS83.S1_s_at	Hypothetical protein	NC	0.99105
88	NC-002229.CDS4.S1_s_at	R-LORF4	NC	0.99105
89	AY510475.CDS4.S1_at	Similar to duck genome sequence	NC	0.99105
90	NC-002229.CDS98.S1_at	SORF1; Hypothetical protein	NC	0.99105
91	AF243438.CDS5.S1_s_at	23KD nuclear protein	0.54	0.11653
92	AF243438.CDS4.S1_s_at	R-LORF7, meq protein (MDV005)	0.76	0.34324
93	NC-002229.CDS5.S1_s_at	R-LORF5	1.4	0.03465

Upper section (1–79): genes that were upregulated in the lytic phase in comparison to latent phase of infection. Middle section (80–90): genes with no change in the expression levels during lytic and latent infection. Lower section (91–93): genes that were up-regulated during the latency infection

[a] Late gene, [b] Immediate Early gene, [c] Early gene (Reviewed in reference 51), [d] NC: No change in the expression level

gB at 5 dpi are significantly higher in comparison to 15 dpi. The low-level expression of vIL-8 and pp38 suggests that these genes may play a role in the establishment/maintenance of latent infection. It is also conceivable that due to the highly cell-associated nature of MDV and lack of a synchronized infection process, the latent infection is comprised a mixed population of MDV particles passing through different stages of infection. The expression pattern of late antigen gB, however, refutes this notion as its expression is hardly detected at 15 dpi ($\Delta\Delta C_T = 7.4 \pm 1.09$). Using in situ hybridization to examine latency-associated transcripts in lymphoid and non-lymphoid tissues of HVT-infected chickens, Holland and Silva [54] have shown that gB is not expressed during the latent phase of infection.

In this study, we examined the transcriptional profiling of MDV genes during lytic and latent infection. Of about 93 genes probed during the productive phase of viral infection, the expression levels of 78 genes were increased from 2.16- to 10.91-fold (Table 3, #1–78). In addition to structural and nucleocapsid proteins, immediate early, early, and even late genes were among the most abundant MDV transcripts expressed during the lytic infection. Other noticeable highly activated genes that play critical roles in the viral pathogenesis, include vIL8, pp38, vLIP, US3, and UL49.5 [Reviewed in reference 3]. The signal intensity of vIL8, a C × C chemokine involved in early cytolytic infection and target cell attraction [55] was 9.36-fold higher than that of latent infection. pp38, A 38 KD phosphoprotein expressed in lytically infected and tumor cells and implicated in reactivation from latency and maintenance of transformation [56–58] was also extensively expressed during the propagation cycle of infection. MDV lipase (vLIP), a homologue of pancreatic lipases, was recently demonstrated to be a virulence factor that enhances viral replication with a potential role in viral pathogenesis [59]. Along with many other virulence-associated MDV genes, vLIP was significantly upregulated during the active propagation phase of viral infection. US3, a serine/therionine protein kinase, and UL49.5, a non-glycosylated envelope/tegument protein, are both implicated in the down-regulation of MHC class I molecules in MDV infected cells [60, 61]. The expression levels of both genes are significantly increased in the lytic phase of infection. UL39 and UL41 are also among the genes with high transcriptional activities in the early phase of MDV infection. Although both genes are known virulence factors for HSV1 [62, 63], their roles in MDV pathogenesis is not well understood. Unpublished results [Silva et al.] indicate that UL41 is probably not a virulence factor in MDV.

The array analysis also revealed the expression pattern of many genes that had no or minimal transcriptional activities during the lytic phase of infection (Table 3, #80–90). The average mean signal intensities of these genes were low (at the level of background noise) and the differences were non-significant (fold change <2 or P value >0.05). It is likely that these genes are encoding for steady-state proteins that are somehow involved in the replication of MDV in vivo and are not affected by the state of infection. If the rate of synthesis of the encoded transcripts matches the rate of degradation, the overall expression levels of the genes will remain unchanged. There were only 3 genes with higher expression values in the latent infection in contrast to lytic phase (Table 3, #91–93), of which only R-LORF5 had statistically significant higher expression value ($P = 0.034$). In a recent study, several splice variants involving meq, vIL8, R-LORF4, and R-LORF5 in the IRL and TRL regions of MDV genome have been identified and characterized [64]. Based on Affymetrix array construct, the probe detecting transcriptional activity of a gene at 1.40-fold change (Table 3, #93) represents R-LORF5. The meq/R-LORF5 splice variant tested in our Real-Time RT-PCR analyses is likely the same R-LORF5 gene product probed in the microarray analysis. The sequence analysis is underway to confirm that the amplicons are meq/RLORF5 splice variant products.

The Affymetrix viral gene microarrays used in this study was constructed based on the original annotation of MDV genome where ORFs encoding LATs were not identified [11, 13]. Although these transcripts were not probed in our array analysis, they were extensively studied and implicated with potential roles in initiation, maintenance, and reactivation of latent infection [21–26]. The latency-associated transcripts also have been identified and characterized in HSV1 (LATs) and VZV (OTF3 and ORF4) with significant roles in establishment and maintenance of latency [65–67]. Cytomegalovirus, a β-herpesvirus, however, has no known viral gene product that might play a role in the induction of latent infection [Reviewed in reference 68].

The in vivo expression pattern of MDV genes was further studied by box and volcano plot analysis. Figure 1 depicts the overall signal intensities of array data with a comparative numerical and statistical expression profiling of MDV genes during both phases of infection and provides a validation for the array results. Figure 2 is a volcano plot depicting fold change versus statistical significance of expression values. As the plot indicates, most viral genes are upregulated during the lytic infection while only a few genes are transcriptionally active during latency (log fold change <0).

In order to further verify the array data, Real-Time RT-PCR was used to analyze the expression pattern of selective MDV genes differentially expressed at 5 and 15 dpi (Table 2). Results indicate that vIL8, pp38, UL41, UL49.5, and gB were significantly upregulated during the lytic phase of infection (89×, 90×, 186×, 145×, 221×, and

169× fold changes, respectively). Array data shows a higher expression levels for meq and R-LORF5 in latent infection (*P* values of 0.34 and 0.034, respectively). The Real-Time RT-PCR results were in good agreement with the array data indicating that both genes had higher transcriptional activities during the latent phase in comparison to lytic phase of infection. The differences observed between the expression values of MDV genes in microarray and Real-Time PCR analysis, is due to the log-based transformed raw data in the array study. Comparative analysis of gene expression between the two phases of infection using raw microarray data, results in similar values as those obtained by Real-Time PCR analysis.

In summary, the expression profiling of MDV transcripts revealed up-regulation of 79 genes during cytolytic infection. This study also provided evidence that three non-LATs-related MDV genes are expressed during both phases of infection. In addition, data obtained from the array analysis indicates that at least 11 known and hypothetical MDV genes are not activated during the active cycle of viral replication. This study provides groundwork for future investigation into the biology and pathogenesis of MDV by generation of mutant strains and identification and characterization of virulence-associated viral genes.

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